ELSEVIER

Journal of Chromatography A, 970 (2002) 167–181

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Surface and wastewater quality monitoring: combination of liquid chromatography with (geno)toxicity detection, diode array detection and tandem mass spectrometry for identification of pollutants

I. Bobeldijk[a,*], P.G.M. Stoks[b], J.P.C. Vissers[c], E. Emke[a], J.A. van Leerdam[a], B. Muilwijk[b], R. Berbee[d], Th.H.M. Noij[a]

[a]*Kiwa Water Research, Groningenhaven 7, P.O. Box 1072, 3430 BB Nieuwegein, The Netherlands*
[b]*Laboratory of the Water Transport Company Rijn-Kennemerland, Groenendael 6, 3449 LV Nieuwegein, The Netherlands*
[c]*Micromass Europe, Transistorstraat 18, 1320 AC Almere, The Netherlands*
[d]*Institute for Inland Water Management and Wastewater Treatment, RIZA, P.O. Box 17, 8200 AA Lelystad, The Netherlands*

Abstract

Identification of unknown water pollutants with liquid chromatography and tandem mass spectrometry (LC–MS–MS) is often more complex and time consuming than identification with gas chromatography and mass spectrometry (GC–MS). In order to focus the identification effort on relevant compounds, unknown peaks need to be selected carefully. Based on its frequency of occurrence in the LC–Diode Array Detection (LC–DAD) chromatograms of surface and infiltrated waters, an unknown peak was selected for identification with LC–MS–MS. This compound was identified as hexamethoxymethylmelamine (HMMM), a chemical often used in the coating industry. This is the first time the presence of this chemical in surface waters has been reported. In addition to HMMM, two other structurally related compounds were found to be present in the investigated surface water. A standard mixture of HMMM and its by-products did not exhibit (geno)toxicity under the test conditions applied in this study. In another example, a genotoxic fraction of an industrial wastewater was isolated and examined by LC–MS–MS using a modern quadrupole–orthogonal acceleration-time-of-flight mass spectrometer (Q-TOF). Four compounds were detected. The structures of two compounds present are proposed to be 9-amino-2-hydroxy-acridine and 9-hydroxy-acridine-*N*-oxide or its structural isomer dihydroxy-acridine. Confirmation with standards could not be carried out, as pure compounds are not available. The other two compounds (structural isomers) could not be identified based on the data available within this study.

Keywords: Water analysis; Genotoxicity detection; Mass spectrometry; Melamine resins; Pollutants; Acridine

1. Introduction

Target compound monitoring (legally binding monitoring programs) with dedicated analytical methods is often insufficient to assess and maintain the quality of surface waters. Many (as yet) unknown compounds can be present, some of them harmful for the environment and also to humans [1]. In particular, in countries where surface water is an important source for drinking water production, the surface water quality needs to be controlled also for the presence of unknown, potentially harmful com-

*Corresponding author. Tel.: +31-30-606-9505; fax: +31-30-606-1165.
E-mail address: ivana.bobeldijk@kiwa.nl (I. Bobeldijk).

0021-9673/02/$ – see front matter
PII: S0021-9673(02)00398-9

pounds. Moreover, also the quality of the main sources of pollution of surface water, e.g. wastewater needs to be the subject of attention. Screening for unknown compounds is often carried out with GC–MS [2], however, if used without derivatisation, this technique is only suitable for non-polar, volatile and semi-volatile compounds. Based on retention times (or retention time indices) and mass spectra (libraries), identification of non-target compounds is performed. After structural elucidation, toxicological or overall risk assessment with respect to drinking water production process, human or environmental health can be performed [3]. For semi-polar and polar target compounds present in water, LC–MS–MS is the technique of choice [4–6]. Although structural elucidation by LC–tandem MS is certainly possible and often applied in the past few years [7,8], it is not as straightforward as with GC–MS. Due to lower sensitivity in full scan mode, the lack of libraries and simple fragmentation interpretation rules, identification with HPLC–MS–MS is time consuming and not always successful. Therefore, rather than performing a general screening for unknown compounds, the identification effort needs to be focused on relevant compounds and not on all unknown peaks present in surface or wastewater extracts. After all, not all LC-amenable compounds present in surface waters are necessarily relevant for man or environment.

One tool for the selection of relevant compounds is HPLC–DAD-fingerprinting [9], as HPLC analysis is often used for the determination of semi-polar, polar or thermally labile target compounds in water samples. With conventional LC–DAD approaches, spectra of all eluting (UV absorbing) organic compounds are acquired, and quite often, the target compounds represent only a part of the total amount of peaks visible in the chromatogram. The additional information is often discarded as being of no interest at that moment. With the approach developed in our laboratory [9], all acquired data are used to obtain extra information on the overall water quality, even without the identity of all peaks being known at that moment. Based on the frequency of their occurrence and apparent concentrations, individual unknowns (characterized by their retention times and UV spectra) can be selected for identification with LC–MS–MS.

Another criterion for prioritizing unknown compounds is the effect they may cause in living organisms [10,11]. The presence of hazardous compounds (e.g. toxicants, carcinogens or endocrine disrupting compounds) in surface waters is undesirable with respect to both human and environmental health. Recently we published a paper describing the combination of the LC–DAD method with effect related detection, the HPLC-ToxPrint method [12]. Unknown genotoxic compounds can be recognized in the HPLC chromatograms of surface water and different types of wastewater by determination of genotoxicity (umu test) in individual LC fractions.

This paper presents two examples of LC–MS–MS identification of unknown compounds, selected by the frequency of their occurrence in the LC–DAD chromatogram or their genotoxicity response in the HPLC-ToxPrint method. The work described here is of qualitative character and is intended to show the possibilities of powerful combinations of different analytical techniques with mass spectrometry in the monitoring of water quality.

2. Experimental

2.1. Chemicals

The HPLC-grade water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Gradient grade acetonitrile was used as the organic modifier (Riedel-de Haën, Seelze, Germany). The eluent was de-aerated using helium (99.999% pure) and placed under a constant pressure of 0.2 bar. HMMM was from ICN Biomedicals (Aurora, OH).

2.2. HPLC–DAD

The samples were filtered over 0.2 μm regenerated cellulose material RC 58 (Schleicher and Schuell, Dassel, Germany). The HPLC–UV-system consisted of a Gilson 232-401 autosampler (Meyvis en co. B.V., Bergen op Zoom, The Netherlands), a gradient HPLC-pump model 250 (Perkin-Elmer, The Netherlands) and a model LC-95 UV-detector (Perkin-Elmer, Gouda, The Netherlands) or a Photo Diode Array detector model 991 (Waters, Etten-Leur, The Netherlands).

The pre-concentration of the sample was carried out on a 20×3 mm I.D. column, packed with OASIS material (Waters). OASIS is a porous co-polymer [poly (divinyl-benzene-co-*N*-vinylpyrrolidone)] with adsorption capacity for both lipophilic and hydrophilic compounds, HLB, 25–35 μm, 73–89 Å pores, 800 m^2/g. The pre-concentration column was mounted on the injection valve of the auto-sampler, and replaced the sample loop.

The analytical column was a 250×4 mm I.D. Inertsil ODS-2, 5 μm material from GL Sciences (Chrompack, Middelburg, The Netherlands). The guard column was 10×2 mm I.D. packed with pellicular C_{18} material, 25–35 μm (Chrompack). The analytical column and the guard column were maintained at a temperature of 8 °C in a column thermostat (W.O. Electronics, Applied Science Group, Emmen, The Netherlands).

The sample was pre-concentrated on-line with a HPLC-pump, the sample volume was 4 ml. The on-line pre-concentration, HPLC separation and fractionation were automated using a column switching system [13]. A linear gradient of acetonitrile (10 to 100%) and water was used, with a flow of 0.7 ml/min (for details, see Refs. [12,13]). Experiments show that the best separation results are obtained if the analytical column is left to equilibrate for 20 min after each analysis (last step of the gradient).

The above described method is used as the basis for all other HPLC–DAD methods (at the participating laboratories) that are used in the data evaluation in this study. Several agreements have been made between the various participants and include the type of analytical column (C_{18} material), pH of the eluent, spectral resolution and the UV spectral range (1.2 nm, 200–350 nm). Two retention time standards were selected for a better comparison of the retention times (see also Results), fenuron and chloroxuron, the gradient is linear between the retention times of these two herbicides.

2.3. HPLC-Toxprint

The sample volume of the industrial wastewater described here was 20 ml. The isolation and separation is identical to the procedure described in Section 2.2. A model 202 fraction collector (Meyvis) is fitted at the outlet tubing of the UV-detector. The fractions 11–46 are collected in a polyethylene 96-well plate, with 1.2 ml/well capacity (Aurora Borealis Control, Schoonebeek, The Netherlands). The fraction collection time is 1 min/well (e.g. compounds eluting between 10 and 11 min are collected in fraction number 11). DMSO is added as a keeper solvent and the HPLC eluent is evaporated under a gentle stream of nitrogen. In each well, the umu test is performed [12]. More details on the HPLC-ToxPrint procedure and the interpretation of the results have been described elsewhere [12].

2.4. Isolation of genotoxic fractions for LC–MS–MS identification

Isolation of distinct (positive) genotoxic fractions is performed as described in Section 2.3. Only the fractions of interest are collected, the overall fractions are discarded. In order to obtain enough material for MS–MS experiments, a larger sample volume is used (100 ml) and the fractionation is performed twice (total of 200 ml). The collected fractions from both fractionations are combined, evaporated and brought to a total volume of 0.4 ml (acetonitrile/water=1:1).

2.5. Off-line extraction of surface water sample

For identification of compounds with LC–MS–MS, an off-line extraction procedure was applied, in order to achieve sufficient concentration of the analytes in the extract. The sample was filtered over 0.2 μm regenerated cellulose material RC 58 (Schleicher and Schuell). Then 500 ml of filtered surface water sample was extracted using Oasis™ solid-phase sorbent. The phase was conditioned with two times 6 ml of methanol and two times 6 ml of Ultrapure water; the sample was loaded onto the column at approximately 2 ml/min, in order to keep the extraction comparable to the on-line method described in Section 2.2. Higher flow-rates can be used if required. After a rinse step with 1 ml of 5% solution of acetonitrile in water and 30 min drying time with nitrogen, the column is eluted with four times 2 ml of acetonitrile. The solvent is evaporated under nitrogen to a final volume of 0.5 ml (1000-fold concentration factor).

2.6. LC–MS–MS experiments

Two types of tandem mass spectrometers were used in this study. A triple stage quadrupole instrument (LC–MS–MS) was used for identification of unknown compounds in surface water and a quadrupole-orthogonal acceleration-time-of-flight instrument (LC-Q-TOF-MS) was used for identification of unknown compounds in the industrial wastewater sample.

2.6.1. LC–MS–MS

The mass spectra were recorded on a Finnigan MAT (San José, CA, USA) TSQ 7000 mass spectrometer using the standard APCI interface of Finnigan MAT. The instrument was tuned in the positive ion mode by infusing several microliters of a 10 mg/l solution of polyethyleneglycol (PEG, Baker) in methanol/water (1:1) with 0.01 *M* of ammonium acetate.

The API interface settings were: heated capillary 150 °C, vaporizer temperature 400 °C, corona needle current 5.00 μA, sheath gas (nitrogen) pressure 75 p.s.i., the auxiliary gas was not used. In full-scan analysis, mass spectra were acquired from m/z 50 up to m/z 800 per s. MS–MS experiments were carried out using argon as the collision gas at a pressure of 2.0 mTorr. Product ion spectra of selected precursors were recorded using alternating collision energies of 5, 20 and 30 eV in order to estimate the optimum collision energy for the observed product ions. In multiple reaction monitoring experiments (MRM), three to four ions were monitored for each compound, including the precursor (at low collision energies, 5 eV) and two to three products (higher collision energies, 20 or 30 eV). Detection was achieved with a multiplier setting of 1900 V. No source fragmentation was used in any of the described experiments.

2.6.2. LC-Q-TOF-MS

The analysis was carried out using a 2690 solvent delivery/sample handling system (Waters) equipped with a 20-μl sample loop and a 250×4 mm I.D. Inertsil ODS-2, 5 μm material analytical column (GL Sciences, Tokyo, Japan). The guard column was 10×2 mm I.D. packed with pellicular C_{18} material, 25–35 μm (Varian-Chrompack, Middelburg, The Netherlands). The LC was interfaced to a Q-TOF2 mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray ionisation source.

The sample was injected onto the analytical column and the compounds were eluted with a flow of 0.7 ml/min using a gradient of 0.1% of formic acid in Ultrapure water and 0.1% of formic acid in acetonitrile (in order to enhance the formation of proton adducts as opposed to sodium adducts during the electrospray ionisation process). This difference in the pH of elution causes a shift in retention times if compared to the experiments described in Sections 2.3 and 2.4. A linear gradient from 10 to 80% (in 40 min) and from 80 to 100% (in 2 min) of acetonitrile was applied. The column was rinsed at a flow of 1 ml/min with 100% acetonitrile for 10 min and then brought to 10% acetonitrile in 2 min. A lock mass was added post-column at a flow-rate of 1–2 μl/min in order to allow for internal mass calibration. For this purpose sulfadimethoxine was used, which ionises well in both positive (m/z 311.0814) and negative (m/z 309.0658) electrospray ionisation mode. The electrospray source conditions in positive electrospray mode were: capillary voltage 3 kV, cone voltage 24 V, source temperature 120 °C. In negative electrospray mode, the conditions were similar, however applying opposite polarities. Pusher frequencies and cycle times were selected automatically. The LC column effluent was introduced into the source of the mass spectrometer without applying any post-column splitting. Hence, 0.7 ml/min of aqueous solvent was continuously introduced into the source of the mass spectrometer, the desolvation gas temperature was set at 450 °C to prevent droplet formation at the probe tip [14]. The resolution of the TOF was set to 7000, in order to enhance sensitivity (in general, the operating resolution of the Q-TOF 2 is 11 000–12 000).

Calibration in positive electrospray mode was conducted with a polyethylene glycol (PEG) mixture containing PEG 200, PEG 400 and PEG 600 in equimolar quantities, the calibration in negative electrospray ionisation mode was performed using rubidium iodide, RbI. PEG and RbI were from Sigma (St Louis, MO, USA).

In order to obtain maximum structural information, the samples were analysed with the MS–MS automatic function switching conditions in centroid mode. In these experiments the survey scan (single MS) was performed in the mass range of 190–500 Da. If required, this range can be broader. In the MS–MS mode, product ion spectra are generated at four collision energies, 15, 20, 25 and 30 eV consecutively, and acquired in the mass range of 50–500 Da. Argon was used as the collision gas and the gas cell pressure in the collision cell was 6×10^{-5} mbar. Acquisition and data processing was performed with MassLynx 3.4 software.

Exact mass of the ions of interest was determined based on the averaged spectra, no background subtraction was needed. In the survey scan, the theoretical mass of the infused sulfadimethoxine was used as the lock mass. In MS–MS spectra, the exact mass of the fragments was determined using the determined exact mass of the precursor as the lock mass. Based on the accurate mass, the (possible) elemental composition of the peaks of interest was calculated using the elemental composition tool within the MassLynx software. Parameter settings were: C 1–50, H 0–100, N 0–10, O 0–10, P 0–2, (S 0–4), even electron ions (for the precursor ions), odd and even electron ions (for the product ions). The appropriate numbers of Cl and Br were determined from the isotopic pattern and added if required. The double bond equivalent (DBE) parameter was set in the default values (−5 to 50) and was not used as an identification criterion, but was used as an indicator of the stability (aromaticity) of the calculated elemental compositions. In this study, the calculated elemental composition possibilities with a maximum deviation of 10 mDa from the measured exact mass were considered. The search was performed in the Merck index, NIST mass spectra library, and the InfoSpec® GC–MS database [2] supervised by Kiwa. The structures found in the databases were evaluated based on the fragmentations observed in the acquired MS–MS spectra. In this process, the ACD (Advanced Chemical Development) MS software manager (available through Micromass) was also used. More details on the experimental procedure and the data processing have been published elsewhere [14].

3. Results and discussion

3.1. HPLC–DAD

3.1.1. General

In order to select unknown compounds relevant for drinking water production in the Netherlands, existing HPLC–DAD data for samples from various locations and laboratories were collected and evaluated. The participating laboratories use HPLC–DAD for the determination of many target pesticides, like phenylurea herbicides. Data files from analyses performed in different periods of the year were selected and included sampling points of raw surface water as well as infiltrated and drinking water.

In order to compare data from different laboratories, retention time indices were calculated as depicted in Eq. (1):

$$Rty_a = Rt_{\mathrm{Fn}} + \frac{(Rt_{\mathrm{Cx}} - Rt_{\mathrm{Fn}})}{(Rt'_{\mathrm{Cx}} - Rt'_{\mathrm{Fn}})}(Rtx_a - Rt_{\mathrm{Fn}}) \quad (1)$$

where Rty_a is the Kiwa retention time index (KRetI) of compound a; Rtx_a, the measured retention time of compound a; Rt_{Cx} and Rt_{Fa}, set KRetI-values of the retention time standards chloroxuron and fenuron; Rt'_{Cx} and Rt'_{Fn}, the measured retention times of the retention time standards chloroxuron and fenuron. The default retention time values of chloroxuron and fenuron were equal to their retention times as measured in our laboratory with the reference HPLC–DAD method (see Experimental). We named the retention time index KRetI (Kiwa Retention time Index). Although not as accurate, this approach is comparable to the Kovats indices used in GC–MS analyses. In a limited round-robin test, the variations of KRetI (as a consequence of the differences in the experimental set-up and the matrix) were determined. Based on the results, a KRetI window of ±1 min was selected as reliable for inter-comparison between laboratories. For every chromatogram, each peak present above a certain threshold (at 215 nm, absorbance > absorbance of chlorotoluron at 50 ng/l) was evaluated. Within the correct KRetI window, spectra were searched in a laboratory-built library of UV spectra and KRetI-values of target compounds and unknown compounds (numbered peak 1 to peak

80). Although the main goal of this process was qualitative information, the concentration of the encountered compounds was estimated by normalizing the peak area with the peak area of chlorotoluron at a concentration of 100 ng/l. This also corrects for the differences in the chromatograms originating from different laboratories. At this stage of the research, the evaluation of the data was performed (partly) manually, but automation of the process is currently being investigated.

3.1.2. Selection of relevant unknown compounds

Sixty chromatograms, originating from eight different water laboratories were evaluated. Based on the frequency and the estimated concentration of their occurrence, all the (known and unknown) compounds were divided into four groups: (I) compounds occurring occasionally and in low concentration; (II) compounds occurring occasionally and in high concentration; (III) compounds occurring frequently and in low concentration and (IV) compounds occurring frequently and in high concentration. The limit for concentration was 250 ng/l, the limit for frequency was two participants or 12 chromatograms. With respect to the overall surface water quality and drinking water production, group IV is of highest relevance [15].

This group, shown in Table 1, contains only two target pesticides, atrazin and diuron. Although peak 10 and peak 25 are not analysed as target compounds at all participating laboratories, these compounds could be identified in the other data as carbamazepine and triphenylphosfineoxide (TPPO) using the data from two of the participants. The other compounds in this group are still unknown. As peak 7 and peak 28 are the most frequently occurring, these two compounds were selected as the priority compounds for identification with GC–MS or LC-tandem MS techniques. In the next step, these relevant unknowns (characterised by their library entry of KRetI and UV spectra) were searched for in the samples analysed within the regular target compound monitoring performed at the participating laboratories. During 2001, peak 28 was not found in concentrations sufficient for identification with LC–MS–MS. Peak 7 was found in two surface water samples, with an estimated concentration around 500 ng/l. Generally speaking, in combination with an enrichment step (1000-fold concentration) this concentration is sufficient for identification with MS–MS using a conventional triple stage quadrupole mass spectrometer.

3.1.3. LC–MS–MS identification of peak 7

A KRetI of about 26 min indicates that this compound is of moderate polarity and should, unless thermally labile, also be amenable to GC–MS. First indication about the identity of peak 7 was indeed obtained from regular GC–MS screening results. In GC–MS analysis data of one of the surface waters, an unknown compound was found, its spectrum showing a good match with the NIST library spec-

Table 1
Compounds occurring frequently and in high concentrations

No. of participants	No. of chromatograms	Highest observed concentration[a] (ng/l)	Peak no./name
3	8	697	Peak 65
3	8	261	Peak 64
2	12	294	Peak 18
3	11	288	Peak 43
3	15	487	Peak 2
5	28	882	Peak 7
6	35	302	Carbamazepine (peak 10)
3	15	539	Atrazine
4	10	859	TPPO (peak 25)
3	18	663	Diuron
4	21	280	Peak 28

Number of participants: 8; number of processed chromatograms: 60.
[a] Estimated concentration, the response was normalised by the response of chlorotoluron at 100 ng/l.

trum of an industrial compound, hexa(methoxymethyl)melamine (HMMM), see Fig. 2. This chemical is predominantly used in the coating industry. A commercially available standard of HMMM was analysed by GC–MS and the identity of the unknown compound was confirmed [16]. HPLC–DAD analysis of the standard showed the presence of several compounds with similar UV spectra. This is in agreement with the literature available on HMMM. It is usually accompanied by complex mixture of compounds formed during the production process [17,18]. The KRetI-values of the components indicated that the most abundant peak in the HPLC chromatogram could be peak 7.

At this stage, confirmation with LC–MS–MS was required. According to the literature and the chemical structure of HMMM, atmospheric pressure chemical ionisation (APCI) in the positive mode is a suitable ionisation technique and was therefore directly used in our experiments. In Fig. 1, a chromatogram of full scan LC–MS analysis of a concentrated standard solution is shown. Many peaks are present in the chromatogram. Extensive studies of similar industrial mixtures have been published elsewhere [17,18] and were outside the scope of this study. For the most abundant compounds, product ion spectra of the protonated molecules were acquired at various collision energies and combined with the information available in the literature. Compounds with retention time (t_R) 23.71 min (molecular mass, MW 376), t_R 28.29 min (MW 390) and t_R 29.64 (MW 420) were identified as penta(methoxymethyl)melamine, HMMM itself and a by-product of double formylation. The product ion spectra and structures are shown in Fig. 2. The less abundant compounds were assumed to be other by-products, with different degrees and combinations of substitutions, as well as several dimeric structures, comparable to those described by Nielen and van de Ven [17] and Chang [18]. Without extensive optimisation of the ionisation and detector conditions, a qualitative MRM method was developed for the compounds with intensities > 10% with respect to the most abundant peak. No quantitative aspects were taken into account. For

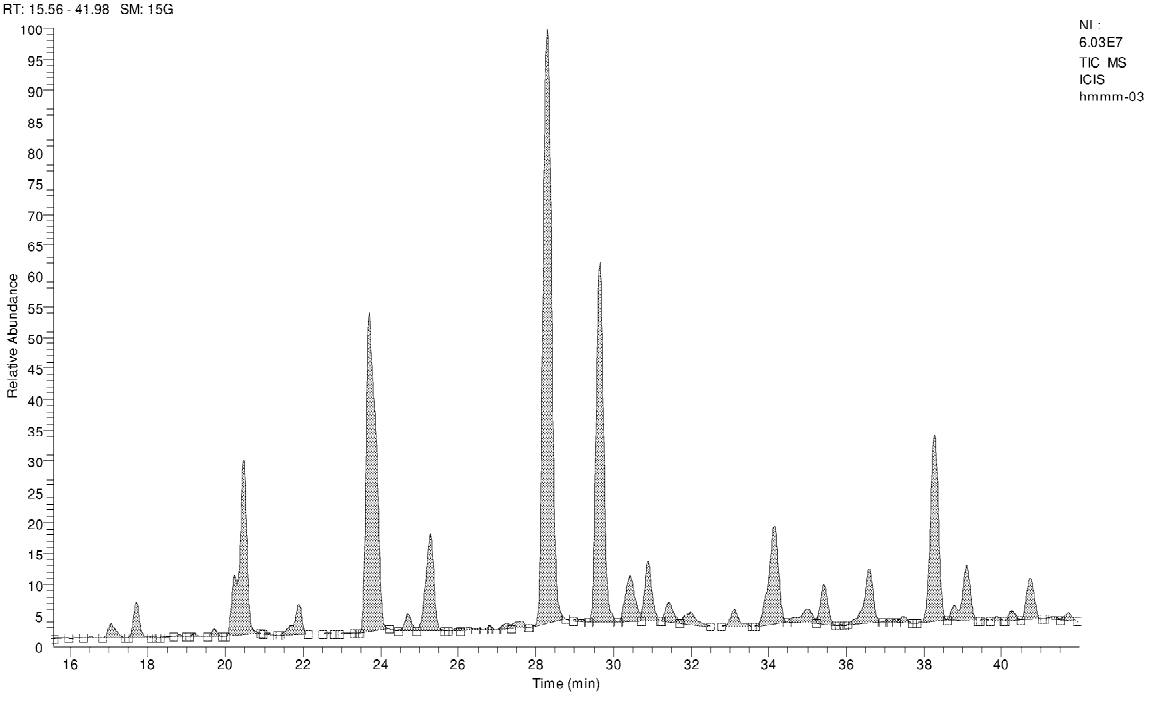
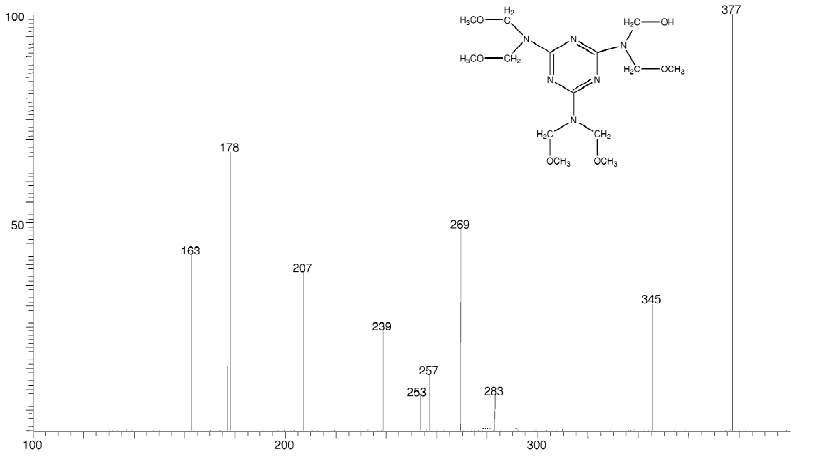
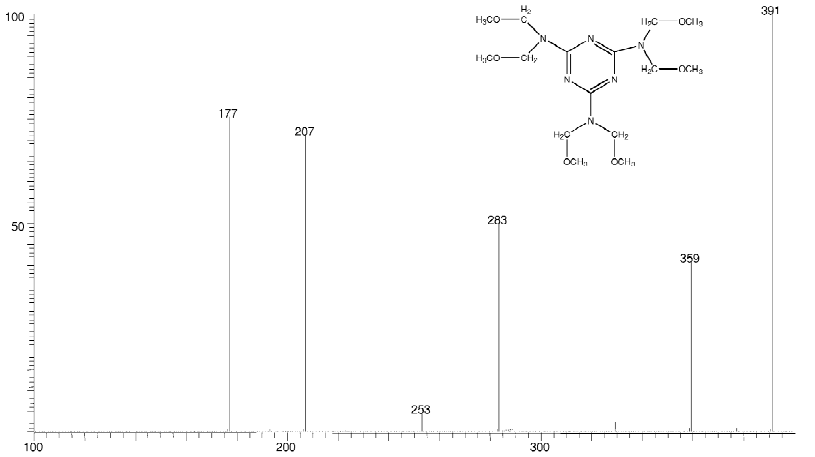


Fig. 1. Chromatogram (part of) of a LC–MS (full scan) analysis of a concentrated standard (1.4 g/l) of HMMM. For conditions, see Experimental.



PMMM



HMMM

Doubly formylated by-product

Fig. 2. Product ion spectra of HMMM, PMMM and the doubly formylated by-product, with structures. The spectra are summed over three collision energies (5, 20 and 30 eV). The fragments in the spectrum are formed by the loss of the individual functionalities.

qualitative purposes three to four specific reactions were monitored for each compound. In combination with retention times, enough specificity is then obtained without considering the relative abundances of the monitored reactions. The surface water extract containing the unknown peak 7, a diluted HMMM standard solution and a procedure blank were analysed. Based on the results, the frequently occurring peak 7 could be identified as HMMM. The blank (not shown) did not contain any of the compounds. In the investigated sample, compounds PMMM, HMMM and the double formylated by-product were present (Fig. 3). The relative abundances of these three compounds in this particular surface water extract differ somewhat from those in the standard (not shown). In the standard, the abundance of compound PMMM is about 5% of that of HMMM and the abundance of the third compound is 20%. In the surface water extract, PMMM is also 5%, however, the abundance of the compound of the doubly formylated by-product is lower, only 10% of the intensity of HMMM. The abundance of the other by-products in the sample is negligible. Based on the abundance of these compounds in HPLC–DAD data, the same ratios were calculated.

The KRetI-values and UV spectra of compound PMMM and the doubly formylated by-product were searched in the existing HPLC–DAD data. In a sample with a high estimated concentration of HMMM (>800 ng/l), a trace of PMMM was found, its spectrum and KRetI were already listed in the library as unknown peak 70. The third compound was not present. In other samples, HMMM and the two structurally related compounds were found in concentrations exceeding 1 μg/l [16].

3.1.4. Toxicity and genotoxicity of the identified compounds

According to the literature, HMMM is of low toxicity to fish (LC_{50} 680 mg/l) [19], more toxicity data could not be found. We therefore tested the standard mix with the umu-test procedure which is usually applied in the HPLC-ToxPrint method (see Experimental). Different dilutions of the standard mix in a range corresponding to 0.05–103 μg/l in the original water sample (assuming a concentration factor of 1000 and a recovery of 100%) were tested without fractionation. The mixture of compounds does not exhibit toxicity nor genotoxicity under the applied experimental conditions. Model experiments showed that HMMM itself is readily removed during the water treatment process applied in the Netherlands (active carbon filters) [16]. The removal of the more polar by-products will be monitored by HPLC–DAD in the follow-up of this study.

3.2. Identification of (genotoxic) compounds in an industrial wastewater

3.2.1. General

The HPLC–DAD technique gives information on the occurrence of individual known and unknown compounds. It does not however provide any information on the toxicological relevance of the (unknown) compounds. For that, the HPLC-ToxPrint was developed. The identification effort can then be directed to harmful compounds. This was applied in investigations of an industrial wastewater. The sample was analysed by the HPLC-Toxprint method [12]. Umu genotoxicity test is carried out in each fraction covering 1-min elution of the HPLC chromatogram, collected in a 96-well plate. The chromatogram and results of the industrial wastewater are shown in Fig. 4. Significant genotoxicity is observed in three distinct fractions: 29, 41 and 43. In this study, fraction 43 was collected and analysed by HPLC-tandem mass spectrometry, using the quadrupole-orthogonal acceleration-time of flight mass spectrometer (Q-TOF) [14,20]. Q-TOF has several advantages (compared to a triple quadrupole MS) which are very important for the identification of unknown compounds: (i) the sensitivity is higher, (ii) accurate mass of all ionised compounds can be determined and used to calculate the possible elemental composition and (iii) under the applied experimental conditions, in most MS–MS spectra, the accurate mass of the fragments can be determined.

3.2.2. Unknown compounds present in the genotoxic fraction

The collected fraction was analysed twice, in positive (ESI+) and negative electrospray (ESI−) ionisation. In both cases, automated MS to MS–MS switching was applied in order to obtain maximum structural information (see Experimental). Three

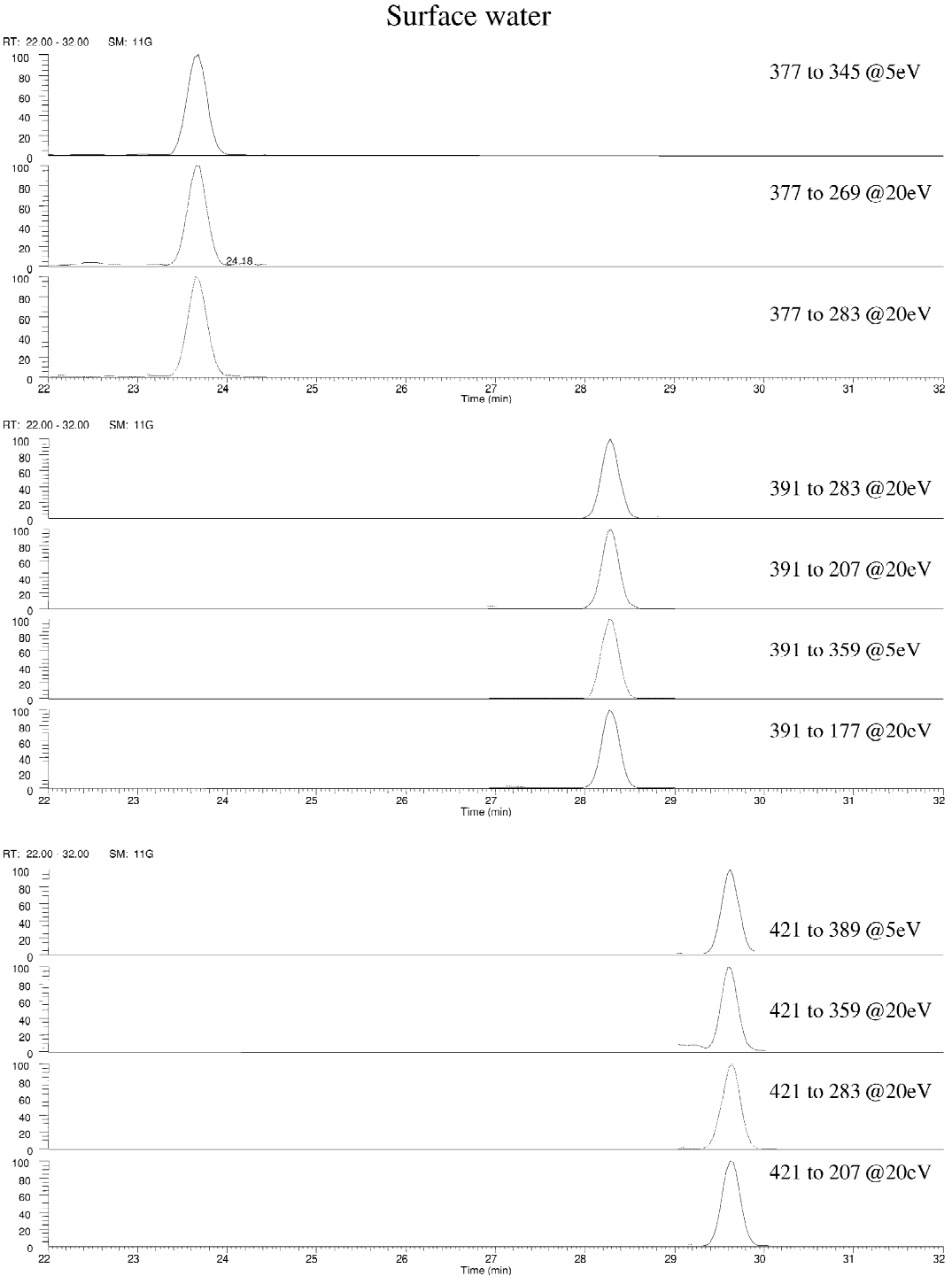


Fig. 3. PMMM (top), HMMM (middle) and doubly formylated by-product (bottom) in a surface water extract.

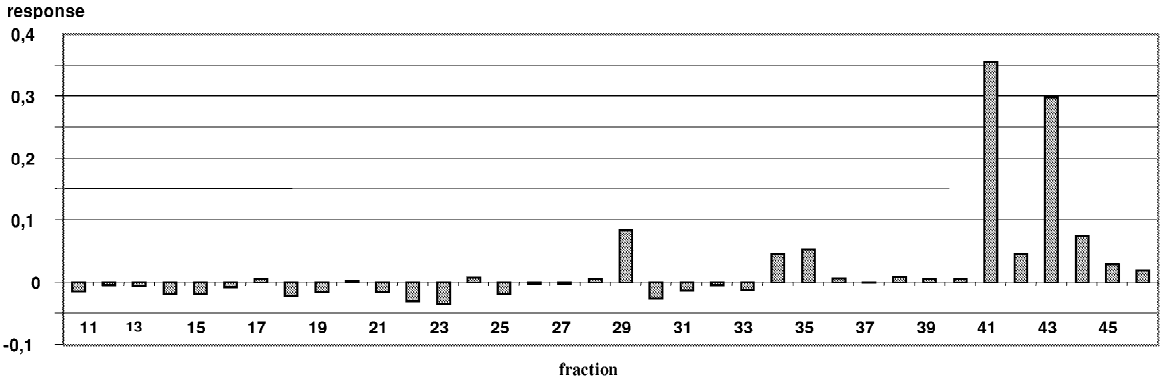


Fig. 4. HPLC-ToxPrint analysis of an industrial wastewater. On the *x*-axis, the fraction number (corresponding to retention time) is given. On the *y*-axis, absorbance at 395 nm is expressed in mV. The zero value of absorbance has been determined as an average of several blanks. In this study, a positive deviation of at least 0.1 mV from the zero value (or the observed trend) is considered a significant (geno)toxicity response.

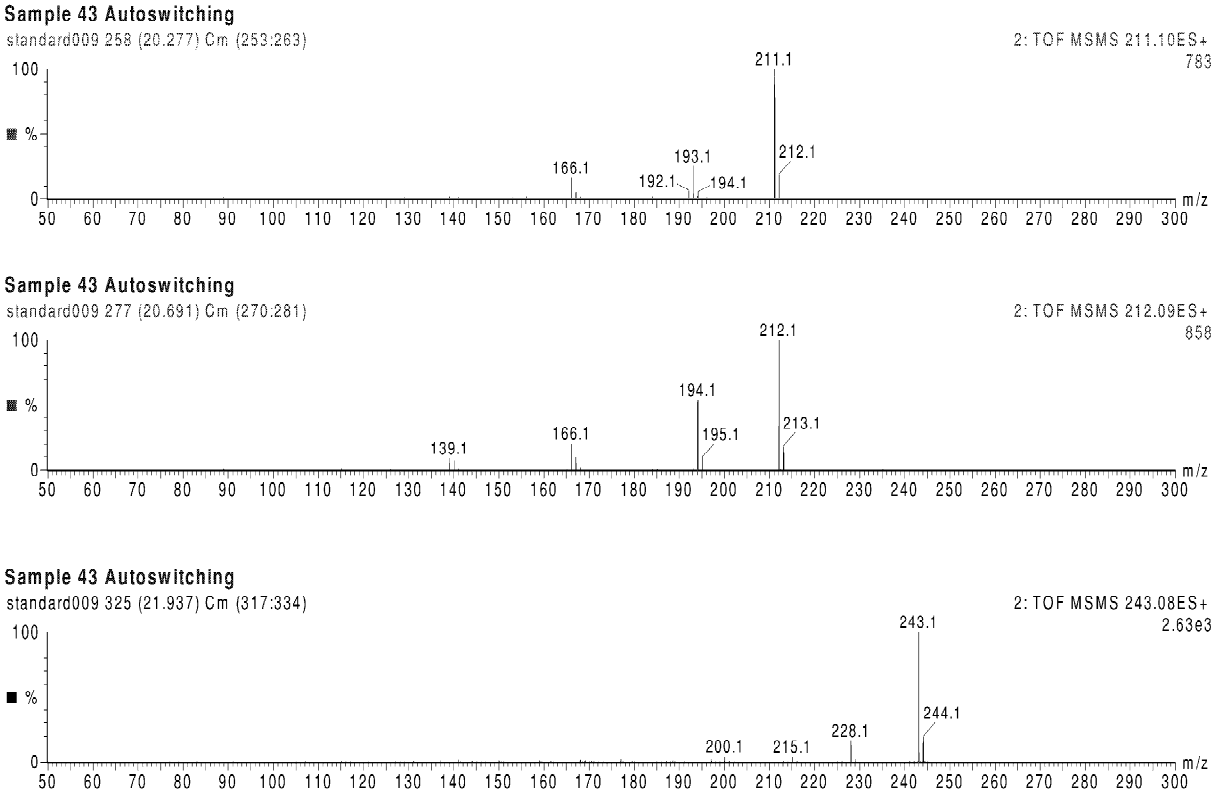


Fig. 5. ESI+ of genotoxic fraction 43: product ion spectra of compounds present. The spectra were summed over four collision energies (15, 20, 25 and 30 eV).

Table 2
Determined accurate mass of the unknown peaks observed in the MS–MS chromatograms of the investigated genotoxic fraction of an industrial wastewater

Retention time (min)	Accurate mass
Positive ionisation mode	$[M+H]^+$
20.3	211.0894
20.7	212.0722
21.9	243.0669
Negative ionisation mode	$[M-H]^-$
20.7	210.0613
21.9	241.0519
22.5	241.0538

compounds with sufficient signal-to-noise ratios can be observed in each MS–MS chromatogram (not shown). The presence of several compounds in one fraction can be explained by the rather broad collection time of each fraction (1 min). The accurate mass of the protonated (ESI+) and deprotonated compounds (ESI−) was determined (Table 2) and used for the calculation of possible elemental compositions. The MS–MS spectra (summed over the various collision energies) are shown in Figs. 6 (ESI+) and 7 (ESI−). By comparing the spectra, it can be concluded that in total four compounds are visible, two of them ionise under both ESI+ and ESI− (MW 211, t_R 20.7 and MW 242, t_R 21.9 min) one in ESI+ (MW 210, t_R 20.3 min) and one in ESI− (MW 242, t_R 22.6 min). Relatively apolar compounds are expected to be resolved in the collected fraction (retention times 42–43 min under the HPLC-ToxPrint conditions).

3.2.3. Unknown compound with MW 211

This unknown compound fragments only at higher

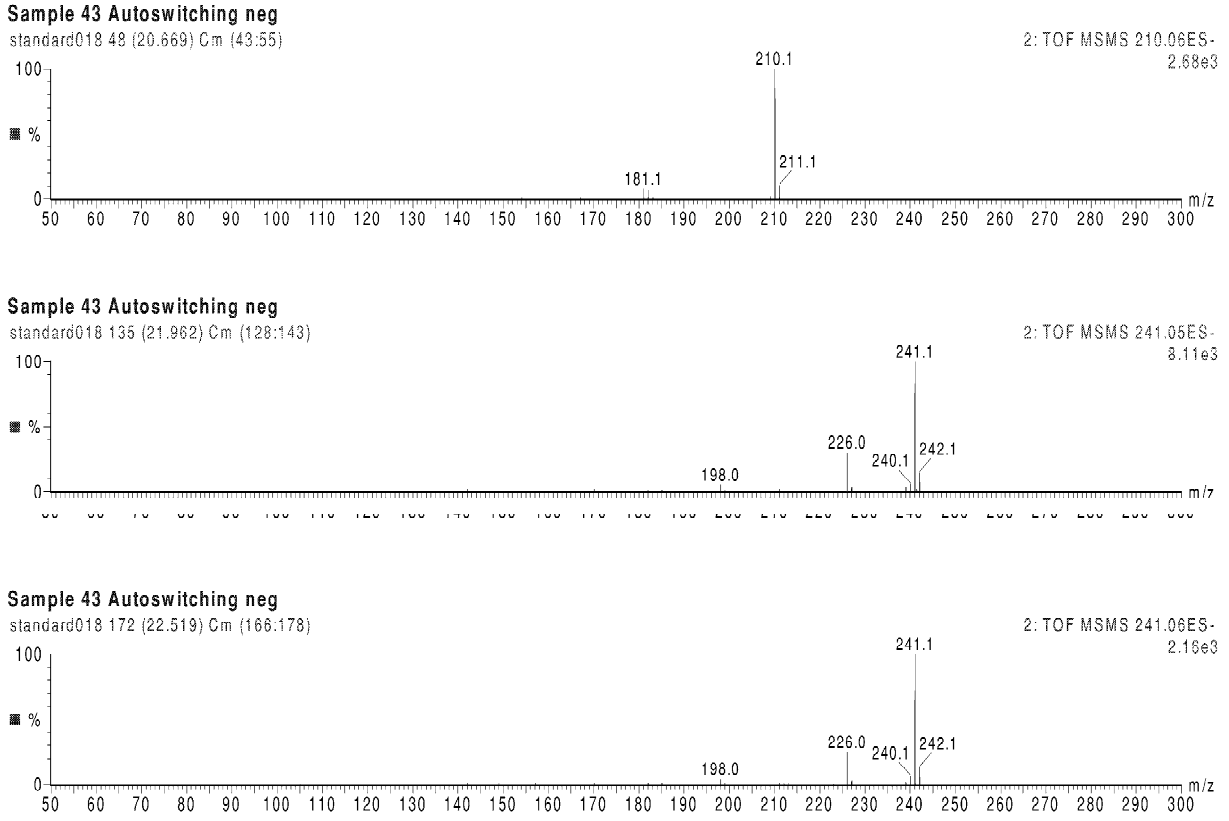


Fig. 6. ESI− of genotoxic fraction 43: product ion spectra of compounds present. The spectra were summed over four collision energies (15, 20, 25 and 30 eV).

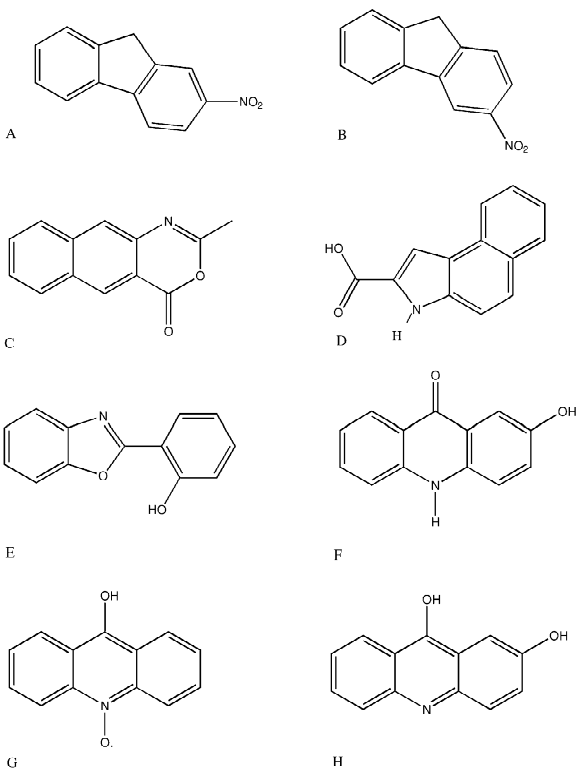


Fig. 7. Unknown compound with MW 211. Evaluated structures for the elemental composition $C_{13}H_9NO_2$. Structures found in the NIST MS library a–g, h enol tautomer of structure f.

collision energies (25 and 30 eV) and at 30 eV the precursor is still present in the product ion spectra with intensities above 40%. Three fragments are formed from the protonated molecule and only one fragment from the deprotonated molecule (Figs. 5b and 6a), all with m/z above 130. This indicates an aromatic compound with a limited number of functional groups. Therefore, only elemental compositions with DBE values higher than six were considered. Two of the chemical formulae fulfil these criteria, $C_{13}H_9NO_2$ (Δ 1.0 mDa) and $C_{13}H_{10}NP$ (Δ 9.3 mDa). The latter did not yield any structures when searched in the available databases; for the first chemical formula, seven structures were found and are shown in Fig. 7a–g. Compounds 7a–c are only expected to ionize under ESI+. From structure 7d, the loss of 28 (ESI−) is difficult to explain, furthermore, this carboxylic acid is too polar to be resolved in fraction 43 under the HPLC-ToxPrint conditions. The presence of other fragments, would be expected in the MS–MS spectra of structure e (e.g. fragments resulting from the cleavage of the bonds in the five-membered ring). The remaining two structures, 7f and 7g can both yield the fragments observed in the product ion spectra. Due to its aromatic structure, 9-hydroxy-acridine-*N*-oxide (10 g) is more stable, than compound 7f. Cleavage of the middle ring of compound 7f is expected at higher collision energies, yielding extra fragments. However, structure 7f can easily form the enol-tautomer 7h, which has an aromatic character and stability comparable to 7g. All the fragments (Fig. 8) formed from the protonated and deprotonated molecule are present also in the electron impact (EI) spectrum of 9-hydroxy-acridine-*N*-oxide, listed in the NIST library (not shown). Under EI fragmentation, fragment 166 is characteristic for many acridine derivatives, which are substituted in the 9-position. However, the mechanism of this fragmentation reaction under the MS–MS conditions described here is unknown. The elemental composition of the fragments is in good agreement with the determined accurate mass of the fragments. In the case of the dihydroxy derivative, the exact position of the second hydroxy group cannot be determined from the available data. We therefore propose that the structure of the unknown compound is either 9-hydroxy-acridine-*N*-oxide or 9,?-dihydroxy-acridine. Unfortunately, standards of these compounds were not available, so the structures could not be confirmed. On the other hand, an

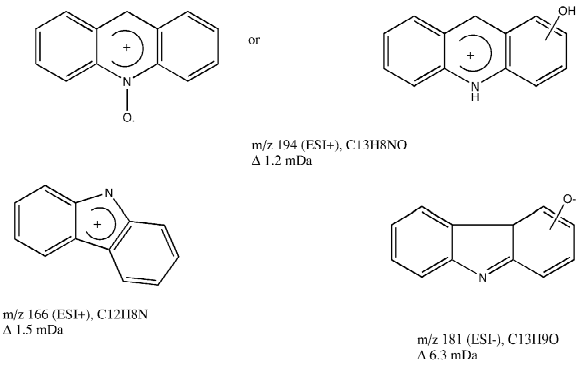


Fig. 8. Proposed structures of ions formed from 9-hydroxy-acridine-*N*-oxide and dihydroxy-acridine. Elucidation of the mechanism of formation of these ions was beyond the scope of this paper and was not studied further.

acridine structure is plausible if the origin of the industrial wastewater is taken into consideration and most acridine derivatives are known to be genotoxic [19] so the acridine can be considered to be the source of genotoxicity in this fraction.

3.2.4. Unknown compound with MW 210 (t_R 20.28 min)

This compound ionises only in ESI+. Elemental compositions $C_{13}H_{10}N_2O$ (Δ 2.2 mDa) and $C_{12}H_{10}N_4$ (Δ −9 mDa) fulfil the criteria of maximum 10 mDa deviation from the determined accurate mass and the high estimated DBE values. The database search yielded 17 structures (not shown), which were evaluated in a similar way to that described above. None of the structures was in agreement with the fragments in the product ion spectra. Surprising is the resemblance of the MS–MS spectrum to that of the acridine derivative described above. The loss of 18 (H_2O) to form an ion with m/z 193 ($C_{13}H_9N_2$, Δ 2.0 mDa) again indicates the presence of a hydroxy functionality. The formation of the ion with m/z 166 ($C_{12}H_8N$, Δ 3.9 mDa) indicates again an acridine structure, substituted in the 9-position with a functionality containing a hetero-atom. Based on this information, the unknown compound was tentatively identified as 9-amino-hydroxyacridine, with elemental composition $C_{13}H_{10}N_2O$. Again, the structural elucidation could not be confirmed with standards.

3.2.5. Unknown compound with MW 242

Based on the accurate mass and the fragments in the MS–MS spectra, it can be concluded that two structural isomers of this compound are present in the collected fraction. The earlier eluting isomer is clearly visible in both ESI+ and ESI−, the latter eluting isomer is better visible in ESI−, even though an MS–MS spectrum with a low signal-to-noise ratio was also acquired under ESI+. In this case, a total of 13 structures (not shown) found in the Merck and NIST databases for elemental compositions $C_{14}H_{10}O_4$ (Δ 1.1 mDa), $C_9H_{10}N_2O_6$ (Δ 5.2 mDa) and $C_8H_{10}N_4O_5$ (Δ −6.1 mDa) were evaluated based on the available information. However, none of these structures could lead to the fragments observed in the MS–MS spectra of the protonated and deprotonated unknown compound(s). The structure of these two isomeric compounds remains unknown.

4. Conclusions

Based on existing HPLC–DAD data for 60 water samples related to drinking water production in the Netherlands, frequently occurring unknown compounds were selected for further investigation. By combining LC–DAD, GC–MS and LC–MS–MS data, one of these compounds was identified as the industrial pollutant HMMM. To our best knowledge, this is the first time the presence of this compound has been reported in surface waters. Two structurally related compounds (by-products from the production process) were identified in the same sample, but in (relatively) lower concentrations. Genotoxicity tests of concentrations relevant for surface and drinking water quality showed that under the applied conditions, the mixture of HMMM and its by-products is not genotoxic nor toxic.

Through effect-related detection, a genotoxic fraction of an LC-chromatogram of an industrial wastewater was detected and isolated. Structural elucidation of the unknown compounds present would not have been possible without using a modern Q-TOF mass spectrometer. Two isomeric compounds could not be identified, two compounds were tentatively identified. The data indicate the presence of acridine derivatives: 9-hydroxy-acridine-*N*-oxide dihydroxy-acridine) and 9-amino-hydroxy-acridine. As in this case, standards of the proposed compounds are not available, the structures could not be confirmed by analysing standard solutions. However, the presence of such compounds is likely in this type of wastewater and also most acridine derivatives are known to be genotoxic.

Applications described in this study show that combination of data from various LC-detection techniques is important in identification of unknown compounds. In water quality monitoring, priority unknowns were selected for identification with LC-tandem MS based on data obtained by LC in combination with DAD and effect-related detection. There is still a great need for libraries containing a large number of MS–MS spectra and even in combination with exact mass (determined by TOF

mass spectrometers), the availability of large databases with structures is necessary to make the identification process more efficient.

Acknowledgements

Part of this study was conducted as part of the Joint Research Programme of the Dutch Waterworks. The authors also wish to thank colleagues at GWA, DZH, PWN and WBB for helpful discussions and for funding the HPLC–UV-fingerprint approach. G.J. de Maagd at Riza is acknowledged for providing the wastewater sample and D. de Zwart at RIVM for help with the search for toxicity data.

References

[1] J. van Genderen, M.N. Mons, J.A. van Leerdam, Inventory and toxicological evaluation of micropollutants. Riwa report, 2000 (in English).
[2] I. Bobeldijk, T. van Leerdam, E. Doomernik, O. Epema, in: E. Gelpi (Ed.), Advances in Mass Spectrometry, Vol. 15, Wiley, Chichester, 2001, p. 929.
[3] Th. Reemtsma, O. Fiehn, M. Jekel, Fresenius J. Anal. Chem. 363 (1999) 771.
[4] I. Bobeldijk, K. Broess, P. Speksnijder, J.A. van Leerdam, J. Chromatogr. A 938 (2001) 15.
[5] R.J.C.A. Steen, I. Bobeldijk, U.A.Th. Brinkman, J. Chromatogr. A 915 (2001) 129.
[6] M.D. Hernando, L. Piedra, A. Belmonte, A. Aguera, A.R. Fernandez-Alba, J. Chromatogr. A 938 (2001) 103.
[7] W.M.A. Niessen, Analusis 28 (10) (2000) 885.
[8] P. Marquet, N. Venisse, E. Lacassie, G. Lachatre, Analusis 28 (10) (2000) 925.
[9] Th.H.M. Noij, E. Emke, Organic Micropollutants in Rhine and Meuse: Monitoring with HPLC–UV-fingerprint, Riwa report, 1999 (in English).
[10] M. Castillo, D. Barcelo, Anal. Chem. 71 (1999) 3769.
[11] Th. Reemstsma, Anal. Chim. Acta 426 (2001) 279.
[12] I. Bobeldijk, A. Brandt, B. Wullings, Th. Noij, J. Chromatogr. A 918 (2001) 277.
[13] Th.H.M. Noij, A. Brandt, in: H.-J. Stan (Ed.), Analysis of Pesticides in Ground and Surface Water. II, Chemistry of Plant Protection, Springer, Berlin, 1995, pp. 91–108.
[14] I. Bobeldijk, J.P.C. Vissers, G. Kearney, H. Major, J.A. van Leerdam, J. Chromatogr. A 929 (2001) 63.
[15] I. Bobeldijk, E. Emke, Organic compounds and water quality: HPLC UV fingerprint, Kiwa report 2000.236 (c), 2000 (in Dutch).
[16] I. Huitema, R. van der Plaat, P.G.M. Stoks, C. Bijsterbosch, H_2O 16 (2001) 7.
[17] M.W.F. Nielen, H.J.F.M. van de Ven, Rapid Commun. Mass Spectrom. 10 (1996) 74.
[18] T.T. Chang, Anal. Chem. 66 (1994) 3267.
[19] www.chemfinder.cambridgesoft.com
[20] M.W.F. Nielen, J.P.C. Vissers, R.E.M. Fuchs, J.W. van Velde, A. Lommen, Rapid Commun. Mass Spectrom. 15 (2001) 1577.